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(54) **Reducible compounds which provide leuco dyes, and analytical compositions, elements and methods containing same.**

(57) Certain reducible compounds are useful in analytical compositions, elements and methods, for example for the detection of bacterial cells. These compounds comprise a moiety which provides a leuco dye upon reduction. This leuco dye can then be oxidized with additional molecules of reducible compound in order to provide a detectable dye. Thus, these reducible compounds are considered bioamplifiers from which a multiplicity of dye molecules can be provided from a single reducible compound molecule. Structurally, the reducible compounds are quinones having suitable substituents which promote varying amounts of leuco dye release at physiological pH.

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REDUCIBLE COMPOUNDS WHICH PROVIDE LEUCO DYES, AND ANALYTICAL COMPOSITIONS, ELEMENTS AND METHODS CONTAINING SAME

This invention relates to clinical chemistry. In particular, it relates to novel reducible compounds and to their use in dry or wet assays of liquids, such as biological fluids, to release useful leuco dyes which can be oxidized to provide dyes. These compounds are especially useful in the detection of microorganisms.

Chemical analysis of liquids, such as water, milk and biological fluids is often desirable or necessary for health maintenance and diagnostic care. Various compositions and elements to facilitate such analyses are known. Such compositions and elements generally include a reagent composition for determining a substance under analysis, identified as an "analyte" herein. The analyte can be a living organism or a nonliving chemical substance. The reagent composition, upon interaction with the analyte, provides a detectable change (for example, dye formation).

Recently, much work has been directed to developing compositions and elements which are useful for rapid and highly quantitative diagnostic or clinical analysis of biological fluids such as whole blood, serum, plasma, urine and the like.

For example, for the rapid and effective diagnosis and treatment of infectious diseases, it is desirable to be able to detect the bacteria causing the disease as rapidly as possible. Infections of the urinary tract are among the most common bacterial diseases, second in frequency only to infections of the respiratory tract. In fact, in many hospitals, urinary tract infections are the most common form of nosocomial infections, often following the use of in-dwelling catheters and various surgical procedures. Most urinary tract infections result from ascending infection by microorganisms introduced through the urethra and vary in severity from an unsuspected infection to a condition of severe systemic disease. Such infections are usually associated with bacterial counts of 100,000 (10^5) or more organisms per ml of urine, a condition referred to as significant bacteriuria. Under normal conditions, urine is sterile, although contamination from the external genitalia may contribute up to 1,000 (10^3) organisms per ml in properly collected and transported specimens.

Significant bacteriuria may be present in a number of pathological conditions involving microbial invasion of any of the tissues of the urinary tract, or may result from simple bacterial multiplication in the urine without tissue invasion. The infection may involve a single site such as the urethra, prostate, bladder, or kidney, although frequently it involves more than one site. Infection restricted to the urine may present itself as asymptomatic bacteriuria, that is, a condition which manifests no overt signs or symptoms of infection. Early treatment of this condition can prevent the development of more serious conditions, for example pyelonephritis (inflammation of the kidney and the renal pelvis). The rapid detection of bacteria by a reliable method would therefore facilitate an early and specific diagnosis.

Further, in order to insure that a prescribed antibiotic is in fact effective in treating an infection, repeated tests during therapy are required. The need for simple, rapid bacteriuria tests is thus clear. Moreover, in view of the frequent unsuspected asymptomatic occurrences of urinary tract infections among children, pregnant women, diabetics and geriatric populations, diagnosis of which may require collection and testing of several specimens, bacteriuria tests must be sufficiently simple and economical to permit routine performance. Again, this illustrates the need for a rapid and inexpensive bacteriuria detection method.

Current laboratory methods based on culturing microorganisms, for example, the calibrated loop-direct streak method, require significant incubation periods (18-24 hours) before results can be determined. These laboratory methods are also time-consuming to perform and require considerable clinical training and facilities.

Known commercial methods for relatively rapid detection of bacteriuria have serious drawbacks. They are tedious, not completely reliable, require complex reagents or instrumentation, and have limited sensitivity to certain microorganisms and susceptibility to drug or other interferences. Hence, the usefulness of known methods is severely limited.

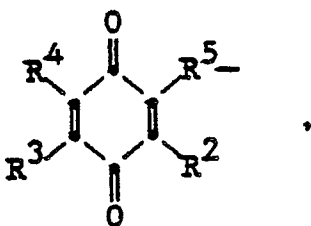
It is also known that colorless materials, for example, tetrazolium salts, can be reduced by microorganisms to form a colored formazan dye. However, the use of formazan dyes for detecting microorganisms has several drawbacks. The formazan dyes generally have low extinction coefficients and therefore cannot be used to detect low levels of microorganisms. The tetrazolium salts have structures are not readily modified to increase the extinction coefficients of the formazan dyes. Some formazan dyes are insoluble in water and can be toxic to the microorganisms.

Multilayer elements for analysis of liquids are also known. Such elements can include an interactive composition which interacts with an analyte to release a preformed, detectable moiety from an immobile carrier nucleus upon oxidation or reduction. Such release generally requires the presence of a highly

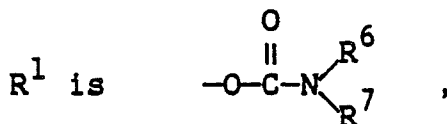
alkaline medium (that is, a pH greater than 13). The spectral absorption band of the preformed detectable moiety is the same before and after release, and radiation-blocking layers are used in the element to screen out unwanted absorption from unreleased detectable moiety during the assay.

EP Publication 0211898 published March 3, 1987 describes reducible compounds which when reduced release detectable moieties, such as dyes. These reducible compounds can be used to detect microorganisms or other analytes which will reduce the compounds at physiological pH (generally a pH from 4 to 9). While providing an advance in the art for detecting such materials, the compounds of Belly et al require one molecule of reducible compound for each molecule of detectable species released. This feature may be sufficient for many analytes which are found in samples in relatively high concentrations. However, where the analyte is present at low concentrations, or where an enhanced signal is required, it would be desirable to have reducible compounds which provide increased signal or can be amplifiable in some manner.

The problems noted above are overcome using a novel reducible compound of the structure CAR-R¹ wherein CAR- is the quinone structure



R² and R⁴ are independently hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted aryl or an electron withdrawing group, R⁵ is substituted or unsubstituted methylene, and R³ is the same as -R⁵-R¹, or is hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted aryl or an electron withdrawing group, or R³ and R⁴, taken together, represent the atoms necessary to complete a substituted or unsubstituted fused carbocyclic ring, the compound characterized wherein



R⁶ is substituted or unsubstituted aryl, and R⁷ is substituted aryl, substituted or unsubstituted anilino, substituted or unsubstituted naphthylimino or an activated methylene group, or R⁶ and R⁷, taken together with the nitrogen atom to which they are attached, represent the carbon and hetero atoms which, after release of R¹ from the quinone nucleus and decarboxylation, form a leuco dye.

The reducible compound noted above can be used in an aqueous composition which is buffered at a pH of 9 or less with one or more appropriate buffers. Alternatively, the compound can be incorporated in a dry analytical element for the determination of an analyte. This element comprises an absorbent carrier material, and one or more reagent zones in which the reducible compound is located.

A method for the determination of an analyte comprises the steps of:

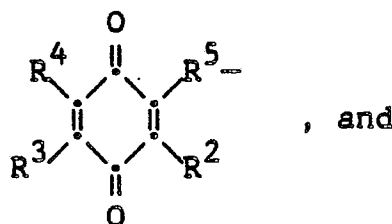
- A. at a pH of 9 or less, contacting a sample of a liquid suspected of containing an analyte with the reducible compound noted above, to provide a leuco dye,
- B. oxidizing the provided leuco dye with additional molecules of the reducible compound to provide a dye, and
- C. detecting the dye provided as a result of the presence of the analyte which effects the release of R¹ from the reducible compound.

The novel reducible compounds of this invention provide the advantage of producing many molecules of dye for each molecule of analyte (that is, for each electron received from an analyte). Such compounds, then can be used to advantage in analytical procedures where the analyte is present in relatively low concentrations, or where amplification is needed to produce a large signal with small amounts of reagents. The compounds of this invention provide these advantages because of the unique R¹ moiety which contains

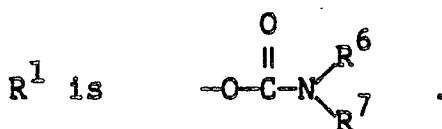
the oxycarbonyl linkage between the quinone nucleus and R⁶ and R⁷ attached to the nitrogen atom. The leuco dye released when the reducible compound is reduced is then oxidized by unreduced reducible compound to provide a desired dye. This cycle, begun with a single electron transfer, of leuco dye formation and subsequent dye formation can be continued as long as there is reducible compound present.

The reducible compounds of this invention are broadly defined as compounds which can be reduced, preferably at a physiological pH (such as from 6 to 9) to release a moiety which becomes a leuco dye (that is, a dye precursor). A molecule of this leuco dye is readily oxidized to provide a dye while acting to reduce a second molecule of reducible compound which begins the cycle again. The net result is a multiplicity of dye molecules from a single electron from an analyte or its reaction products.

More particularly, the compounds of this invention are defined by the structure CAR-R¹ wherein CAR- is the quinone structure



, and



In the quinone nucleus illustrated above, R² and R⁴ may be independently (that is, the same or different) hydrogen, substituted or unsubstituted alkyl of 1 to 40 carbon atoms (for example methyl, ethyl, hydroxymethyl, methoxymethyl, benzyl and others apparent to one skilled in the art) substituted or unsubstituted aryl (for example phenyl, naphthyl, methylnaphthyl, *p*-nitrophenyl, *m*-methoxyphenyl, phenyl-sulfonamido and others apparent to one skilled in the art) or an electron withdrawing group which generally has a positive Hammett sigma value, and preferably has a sigma value greater than 0.06. Hammett sigma values are calculated in accordance with standard procedures described, for example in Steric Effects in Organic Chemistry, John Wiley & Sons, Inc., 1956, pp. 570-574 and Progress in Physical Organic Chemistry, Vol. 2, Interscience Publishers, 1964, pp. 333-339. Representative electron withdrawing groups having positive Hammett sigma values include cyano, carboxy, nitro, halo (fluoro; bromo, chloro or iodo), trihalomethyl (for example trifluoromethyl, or trichloromethyl), trialkylammonium, carbonyl, carbamoyl, sulfonyl, sulfamoyl, esters and others readily apparent to one skilled in the art, or alkyl or aryl groups (defined above) substituted with one or more of these electron withdrawing groups. Preferred electron withdrawing groups include *p*-nitrophenyl, *m*-nitrophenyl, *p*-cyanophenyl and 2,5-dichlorophenyl. Aryl groups with methoxy or acetamido groups in the meta position are also useful.

R³ is the same as -R⁵-R¹, or it is hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted aryl or an electron withdrawing group as defined above for R² and R⁴. Alternatively, R³ and R⁴, taken together, represent the atoms necessary to complete a substituted or unsubstituted fused carbocyclic ring attached to the quinone nucleus. For example, such a ring (mono- or bicyclic) can have from 4 to 8 carbon atoms in the backbone.

R⁵ may be methylene which is unsubstituted or substituted with methyl or methoxy.

R⁶ may be a substituted or unsubstituted aryl of 6 to 10 carbon atoms, such as phenyl, 4-aminophenyl, naphthyl, *p*-dimethylaminophenyl, *p*-hydroxyphenyl or 4-dimethylaminonaphthyl and others known to one skilled in the art.

R⁷ may be aryl of 6 to 14 carbon atoms in the nucleus which is substituted with substituents which provide with the nitrogen atom a leuco dye moiety (such as hydroxy and tertiary amino), or it can be substituted or unsubstituted anilino, substituted or unsubstituted naphthylimino (such as anilino, *p*-nitroanilino, naphthylimino or 4-sulfonylmethylnaphthylimino), or an activated methylene group (such as α -benzoylacetanilide).

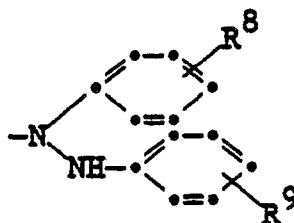
Alternatively, R⁶ and R⁷ taken together with the nitrogen atom to which they are attached, represent the carbon and hetero (nitrogen, oxygen, selenium, sulfur, phosphorus) atoms which, after release of R¹ from

the quinone nucleus and decarboxylation, form a leuco dye. Such leuco dye moieties (that is, R^6 , R^7 with the nitrogen atom) are reduced forms of dyes, such as leuco azo dyes, leuco azomethine dyes, leuco azoanilines or diarylamine compounds.

Representative examples of useful $-NR^6R^7$ groups include, but are not limited to, the following:

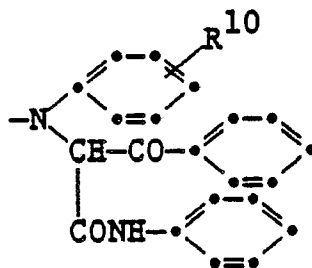
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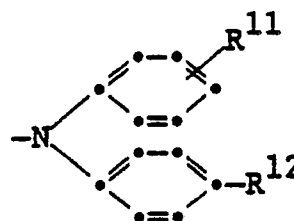
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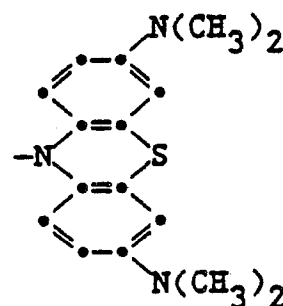
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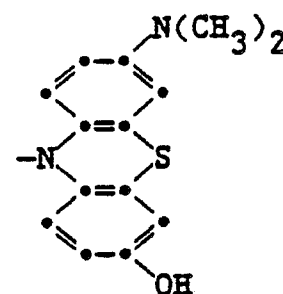
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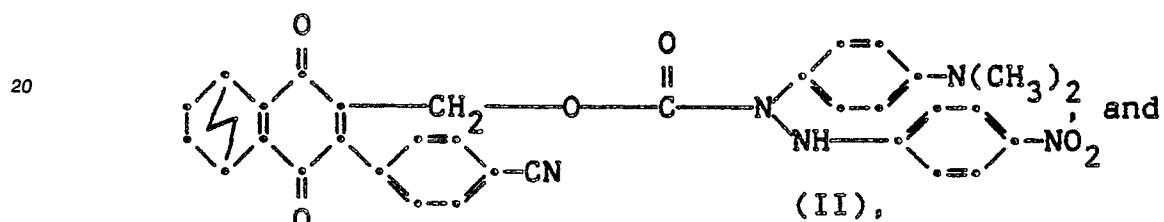
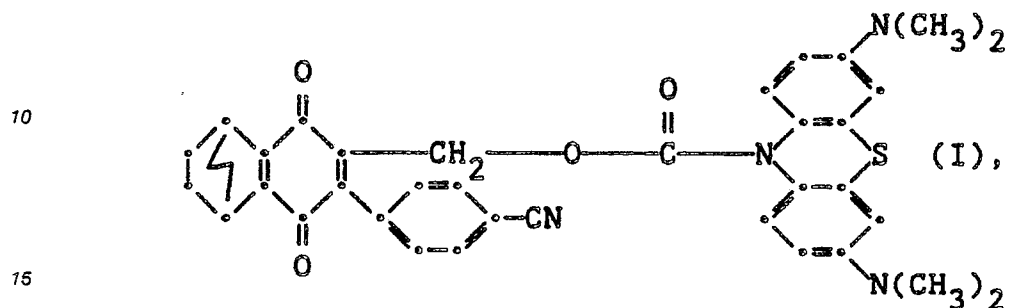


wherein R^8 , R^9 , R^{10} and R^{11} are independently of each other any of the substituents such as nitro, alkylsulfonyl (of 1 to 3 carbon atoms), tertiary amino, alkoxy (of 1 to 3 carbon atoms), alkyl (of 1 to 3 carbon atoms), and others known to one skilled in the art), and R^{12} is hydroxy or tertiary amino.

When the reducible compound of this invention is reduced, the R^1 moiety is released. Since this moiety contains an unstable carbamic acid linkage, it spontaneously undergoes decarboxylation to form a leuco dye.

The preferred reducible compounds of this invention have at least two electron withdrawing groups for R^2 , R^3 and R^4 as defined above, or R^3 and R^4 , taken together, represent the atoms necessary to complete a substituted or unsubstituted fused 5- to 7-member carbocyclic ring as defined above. More preferably, R^5 is unsubstituted methylene and R^3 and R^4 are the 5- to 7-membered carbocyclic ring as defined above.

5 Representative reducible compounds of this invention include, but are not limited to:



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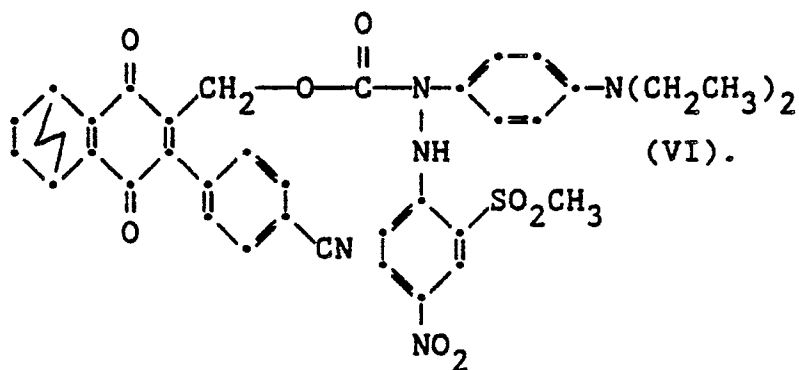
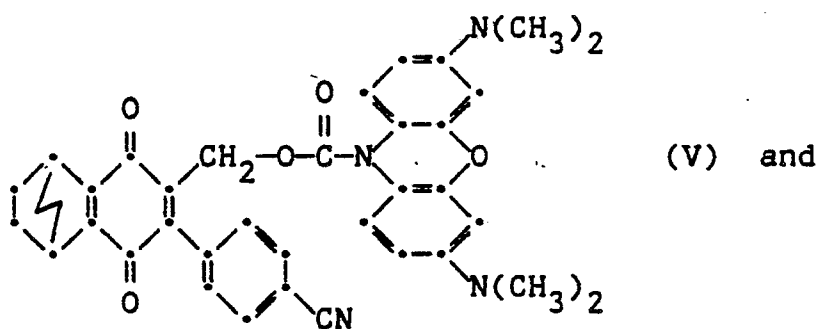
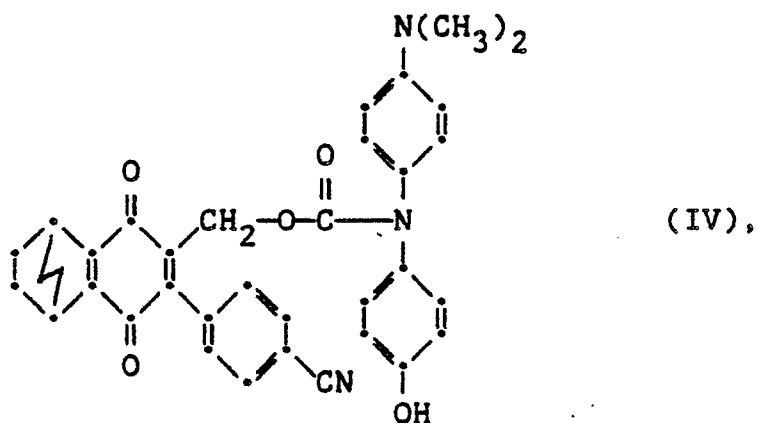
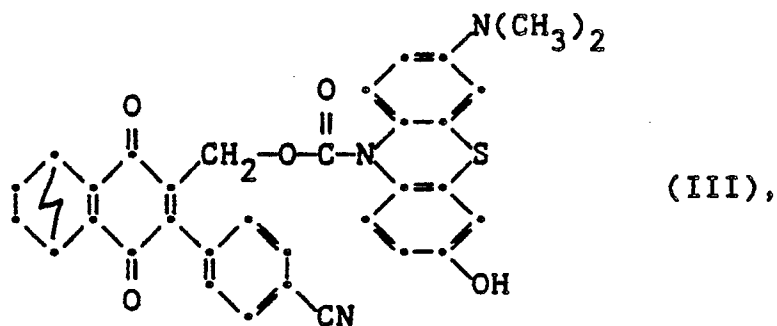
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Compound I is preferred.

The novel reducible compounds of the present invention are prepared using a sequence of individually known reactions. Generally, the preparation sequence includes the following general steps: (1) preparation of the eventual dye, (2) hydrogenation of the dye to a leuco dye, (3) phosgenation of the leuco dye and (4) reaction of the leuco dye carbamyl chloride and a hydroxymethyl CAR-derivative. Representative preparations are provided in Examples 1 and 2 below.

Generally, the reducible compounds described herein have limited water solubility. Hence, it is best,

when using them in an aqueous environment, to prepare a dispersion of the compound prior to use. Such dispersions generally comprise the reducible compound, an aqueous buffer solution and either a water-solubilizing surfactant or a water-miscible organic solvent for the compound, or both.

Surfactants which are useful in the practice of this invention include any surfactants which do not inhibit compound reduction. Generally, for detection of living cells, the useful surfactants are nonionic surfactants, including, for example, alkylaryl polyethoxy alcohols (for example, Triton™ X-100 and X-305), p-alkylaryl oxypolyglycidols (for example, sold under the trade name Surfactant 10G), Tween 80 and others known to one skilled in the art.

Useful water-miscible organic solvents include, but are not limited to, alcohols (for example, methanol, ethanol, propanol and others known in the art), N,N-dimethylformamide, dimethyl sulfoxide, acetonitrile or hexamethylenephosphoramidate. The particular solvent to be used for a particular reducible compound can be readily determined by routine experimentation.

A dispersion can be prepared in the following general manner with the particular details of such a preparation illustrated in Example 3 below. The reducible compound is dissolved in the water-miscible solvent at a concentration which depends upon its molecular weight, but generally at from 1 to 100, and preferably from 5 to 80, mg per ml of solvent. The resulting solution is then mixed with a suitable surfactant in an amount generally of from 0.1 to 24, and preferably from 0.5 to 10, mg surfactant per ml of dispersion. This preparation is generally carried out at room temperature.

These dispersions generally contain a buffer in an amount effective to preferably maintain a physiological pH (such as 6 to 9). The concentration of buffer in the dispersion can vary widely, but is generally from 0.01 to 0.1 molar. Representative buffers include phosphates, borates and others reported by Good et al in *Biochemistry*, 5, 467 (1966), and *Anal. Biochem.*, 104, 300 (1980).

The reducible compounds described herein are useful in compositions for analytical determination (that is, qualitative or quantitative detection) of aqueous and nonaqueous liquids, for example, biological fluids, manufacturing processes, wastewater or food stuffs. Determinations can be made of various analytes using a single reaction or a sequence of reactions which bring about reduction of the compound and provision of the leuco dye. The various analytes include living cells (for example, bacteria, white blood cells, yeast or fungi), enzymes (for example, lipase, glucose oxidase, lactate oxidase, creatine kinase, α -glycerophosphate oxidase, lactate dehydrogenase, pyruvate dehydrogenase, glucose-6-phosphate dehydrogenase, alanine aminotransferase, aspartate aminotransferase and other NADH-based, FADH-based or oxidase-based assays), biological or chemical reductants other than living cells which will reduce the reducible compound (for example, ascorbates, cysteine, glutathione or thioredoxin), metabolizable substances (for example, glucose, lactic acid, triglycerides or cholesterol) and immunoreactants (for example, antigens, antibodies or haptens).

The compositions can be used to monitor enzyme redox reactions as well as flavin adenine dinucleotide (FAD-FADH)-based and nicotinamide adenine dinucleotide (NAD-NADH)-based and (NADP-NADPH)-based reactions. In such instances, the reducible compound can be used to provide a dye in place of NADH.

The reducible compounds of this invention, are particularly useful in detecting or quantifying living cells in biological samples. Although any biological sample suspected of having living cells therein can be analyzed for bacteria, white blood cells, yeast or fungi by this invention, the invention is particularly useful for bacterial detection in biological fluids, such as human and animal fluids (for example, urine, cerebral spinal fluid, blood as well as stool secretions) and suspensions of human or animal tissue. The practice of this invention is particularly important for detection of urinary tract infections in urine (diluted or undiluted).

When determining living cells using the reducible compounds, it is preferable that the living cells interact with an electron transfer agent (identified herein as ETA). The presence of an ETA may also provide more efficient dye release for analytical determinations of nonliving analytes. The ETA is a mobile compound which acts as an intermediary between the substance being determined (for example, living cell) and the reducible compound.

In general, the potential of the ETA should be more positive than the potential of the substance to be determined and less positive than the potential of the reducible compound of this invention. That is, the ETA should be more easily reduced than the analyte and less easily reduced than the reducible compound. They are generally present at a concentration that is dependant upon the concentration of the analyte, and preferably at a concentration of from 1×10^{-3} molar to 1×10^{-7} molar.

ETA compounds useful in the practice of this invention include phenazine methosulfate, phenazine ethosulfate and similar compounds known to one skilled in the art. Combinations of different ETA compounds can be used if desired. Preferred ETA compounds useful in the practice of this invention are described in U.S. Patent 4,746,607.

The detection of living cells, and particularly of bacterial cells, is often carried out in the presence of a

nutrient for those cells although its presence is not essential. Any nutrient media can be used which contains useful carbon, and optionally nitrogen, sources. Suitable nutrient media having proper components and pH are well known in the art. Particularly useful nutrients are glucose or tryptose alone or in combination.

- 5 The present invention is adaptable to either solution or dry assays. In a solution assay, a solution (or dispersion) containing a reducible compound, and preferably an ETA, is prepared and contacted with a liquid test sample containing the living cells or analyte to be determined by mixing. The ETA can also be mixed with the test sample prior to mixing with the reducible compound. Generally the reducible compound is mixed with the test sample in a suitable container (for example, test tube, petri dish beaker or cuvette).
- 10 The resulting solution (or dispersion) is gently mixed and incubated for a relatively short time at a temperature up to 40 °C. The test sample is then evaluated by measuring the dye at a suitable wavelength. Such an evaluation can be done with suitable detection equipment.

- A solution assay can also be carried out by contacting a porous, absorbent material, for example a paper strip, containing the test sample with a dispersion of the reducible compound. The analyte in the test sample can migrate from the porous material into the dispersion and initiate the analytical reactions needed for determination. In solution assays, the amount of reducible compound present is at least 0.001, and preferably from 0.01 to 1.0, millimolar. Other reagents can be present in amounts readily determined by one skilled in the art.

- Alternatively, the method of this invention can be practiced in a dry assay with a dry analytical element.
- 20 Such an element can be an absorbent carrier material, that is, a thin sheet or strip of self-supporting absorbent or bibulous material, such as filter paper or strips, which contains the reducible compound or a dried residue of the dispersion comprising same. Such elements are known in the art as test strips, diagnostic elements, dip sticks, diagnostic agents and the like.

- When employed in dry analytical elements, the reducible compounds described herein can be incorporated into a suitable absorbent carrier material by imbibition or impregnation, or can be coated on a suitable absorbent carrier material. Alternatively, they can be added to the element during an assay. Useful carrier materials are insoluble and maintain their structural integrity when exposed to water or biological fluids such as urine or serum. Useful carrier materials can be prepared from paper, porous particulate structures, cellulose, porous polymeric films, wood, glass fiber, woven and nonwoven fabrics (synthetic and nonsynthetic) and the like. Useful materials and procedures for making such elements are well known in the art as exemplified by U.S. Patents 3,092,465, 3,802,842, 3,915,647, 3,917,453, 3,936,357, 4,248,829, 4,255,384 and 4,270,920, and U.K. Patent 2,052,057.

- A dry assay can be practiced to particular advantage with an analytical element comprising a nonporous support having thereon at least one porous spreading zone as the absorbent carrier material.
- 35 The reducible compound can be in the spreading zone or in a different zone (for example, reagent zone, registration zone or hydrophilic zone). The spreading zone can be prepared from any suitable fibrous or non-fibrous material or mixtures of either or both. The void volume and average pore size of this zone can be varied depending upon the use intended. For example, if whole blood or other liquid samples containing cells or high molecular weight materials are to be assayed, the void volume and average pore size are generally greater than if serum or urine is to be assayed.

- 40 The spreading zone can be prepared using fibrous materials, either mixed with a suitable binder material or woven into a fabric, as described in U. S. Patent 4,292,272, from polymeric compositions (for example, blush polymers) or particulate materials, with or without binding adhesives, as described in U. S. Patents 3,992,158, 4,258,001 and 4,430,436, and Japanese Patent Publication 57(1982)-101760. It is desired that the spreading zones be isotropically porous, meaning that the porosity is the same in each direction in the zone as created by interconnected spaces or pores between particles, fibers or polymeric strands.

- The dry analytical element of this invention can be a single self-supporting porous spreading zone containing a reducible compound and any other desired reagents for a particular use, but preferably such zone is carried on a suitable nonporous support. Such a support can be any suitable dimensionally stable, and preferably, transparent (that is, radiation transmissive) film or sheet material which transmits electromagnetic radiation of a wavelength between 200 and 900 nm. Useful support materials include polystyrene, polyesters, polycarbonates and cellulose esters.

- 55 The elements can have more than one zone which are generally in fluid contact with each other, meaning that fluids, reagents and reaction products can pass between superposed regions of adjacent zones. Preferably, the zones are separately coated superposed layers, although two or more zones can be in a single coated layer. Besides the patents noted above, suitable element formats and components are described also, for example, in U. S. Patents 4,042,335 and 4,144,306 and U.S. Reissue 30,267.

In the elements of this invention, the amount of the reducible compound can be varied widely, but it is

generally present in a coverage of at least 0.01, and preferably from 0.05 to 0.2, g/m². Optional reagents are generally present in the following coverages:

ETA:	generally at least 0.001, and preferably from 0.01 to 1, g/m ² ,
nutrient:	generally at least 0.05, and preferably from 0.1 to 2, g/m ² (used only in living cell detection),
buffer (pH $\leq$ 9):	generally at least 0.1, and preferably from 0.5 to 2, g/m ² , and
surfactant:	generally at least 0.1, and preferably from 0.2 to 5, g/m ²

One or more of the zones can contain a variety of other desirable, but optional, components, including activators, binders (generally hydrophilic), antioxidants, coupler solvents, etc. as is known in the art, as well as any reagents needed for assay of a particular analyte.

In one embodiment of this invention, an element for detection of microorganisms in an aqueous liquid comprises an electron transfer agent and a reducible compound, both of which are described above. It is desirable that these elements also contain a nutrient for the living cells and a buffer which maintains physiological pH during the assay. Such an element can be used to detect bacteria, for example, in a urine sample (for example, one pretreated to eliminate reductive interferences) by physically contacting the sample and element in a suitable manner, and detecting the detectable species released from the reducible compound as a result of the presence of the bacteria at the appropriate wavelength.

In another embodiment of this invention, an element is used for the determination of a nonliving biological or chemical analyte in an aqueous liquid. An interactive composition containing one or more reagents can be incorporated into the element or added at the time of the assay. This composition contains the reagents needed to interact with the analyte to bring about reduction of the reducible compound and eventual dye formation. Examples of such analytes are described above. The amount of dye detected can be correlated to the amount of analyte present in the liquid sample.

The element of this invention is also useful for determining other reductants such as ascorbate (ascorbic acid and equivalent alkali metal salts), cysteine, glutathione, thioredoxin and the like.

A variety of different elements, depending on the method of assay, can be prepared in accordance with the present invention. Elements can be configured in a variety of forms, including elongated tapes of any desired width, sheets, slides or chips.

The assay of this invention can be manual or automated. In general, in using the dry elements, an analyte or living cell determination is made by taking the element from a supply roll, chip packet or other source and contacting it with a sample (for example, 1-200 μ l) of the liquid to be tested so that the sample mixes with the reagents in the element. Such contact can be accomplished in any suitable manner, for example, by dipping or immersing the element into the sample or, preferably, by spotting the element by hand or machine with one or more drops of the sample with a suitable dispensing means.

After sample application, the element is exposed to any conditioning, such as incubation, heating or the like, that may be desirable to quicken or otherwise facilitate obtaining any test result.

Detection of an analyte or living cell is achieved when the reducible compound is reduced releasing a leuco dye which in turn is oxidized by another molecule of reducible compound to provide a dye which can be detected in a suitable manner. Spectral determinations can be made either at the maximum wavelength of the dye or at wavelengths other than the maximum wavelength.

Escherichia coli (ATCC 25922) cells were grown in BHI medium at 37° C without shaking and transferred daily. Forty ml of the cells grown overnight were harvested by centrifugation and resuspended in 10 ml of a 0.05 molar potassium phosphate buffer (pH 7.5). Solution 1 was prepared by adding 5 ml of the cell suspension to 9 ml of buffer. Solution 2 was prepared by adding 1 ml of solution 1 to 9 ml of buffer. Solutions 2a, 2b, 2c, and others, were prepared by a 1:1 dilution of Solution 2. The turbidity of each solution was measured at 620 nm against a buffer blank in a commercially available Beckman A25 spectrophotometer. A linear relationship between turbidity measurement and viable cell counts had been predetermined. An absorbance of 0.1 was found to be equivalent to 6×10^7 *E. coli* cells/ml. Using this relationship and the known dilution factor, the number of cells in Solution 1 was determined.

The following examples are presented to illustrate the practice of this invention.

Example 1: Preparation of Compound I

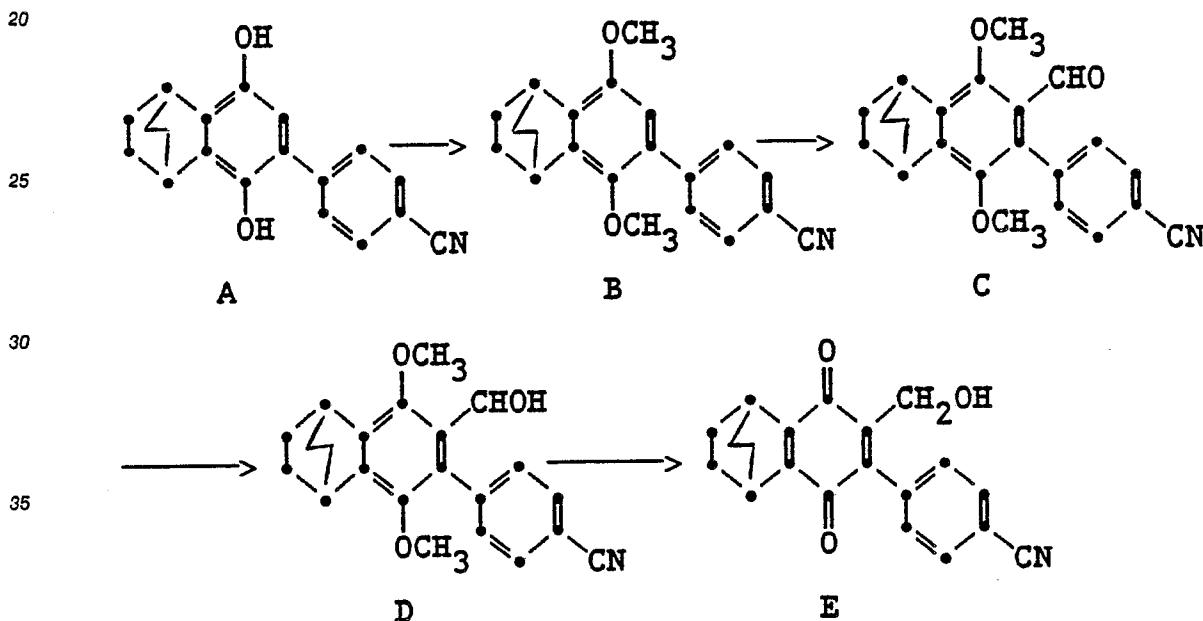
Compound I identified above was prepared as follows:

Preparation of Leuco Methylene Blue Carbamyl Chloride Intermediate:

Methylene blue (5.5 g, 0.017 mole) was mixed with 2,6-lutidine (5 ml) and palladium catalyst (200 mg, on carbon) in dry acetonitrile (200 ml) in an hydrogenation bottle under hydrogen pressure. This mixture was hydrogenated with vigorous shaking at 3 atmospheres hydrogen pressure until a colorless solution was obtained. The resulting solution was rapidly filtered into a stirred solution of phosgene (20 ml of a 1 molar solution in toluene) and dry acetonitrile (50 ml) while maintaining a nitrogen atmosphere over the solution. A white solid precipitated, was collected, washed with acetonitrile and dried to give 2.6 g of intermediate.

Preparation of Hydroxymethyl Quinone Intermediate:

The following reaction sequence was followed to prepare this intermediate.



Compound A (identified above) was prepared by the procedure described in EP Publication 0211898, Example 21 and Example 1 (Steps 2a, 2b and 3).

Compound A (25.2 g, 86.4 mmole), iodomethane (49 g, 346 mmole) and potassium carbonate (35.8 g, 259 mmole) were heated at reflux in acetone (150 ml) for 24 hours. The reaction mixture was allowed to cool and then poured into dilute hydrochloric acid in ice/water (200 ml). The precipitated solid was collected by filtration and washed with water, air dried and recrystallized from ethanol with decolorizing carbon to give 16.75 g (60.6% yield) of Compound B (m.p. 186-187 °C). The structure (identified above) was confirmed by NMR.

Titanium (IV) chloride (38 g, 0.2 mole) was added to an ice cold solution of Compound B (32 g, 0.1 mole) in dichloromethane (750 ml), stirring under a nitrogen atmosphere. To this solution was added dropwise α,α -dichloromethylmethylether (18.9 g, 0.165 mole), and the reaction mixture was allowed to warm to room temperature over about 15 hours. The mixture was again cooled and ice water (1 liter) was carefully added dropwise. The resulting layers were separated and the water layer was washed with dichloromethane. The combined organic layers were dried and the solvent was removed. The crude product was purified by chromatography (silica; dichloromethane) and the resulting solid was triturated in cold diethylether, filtered and washed with cold diethylether to give Compound C (16.1 g, 46.5% yield) as a white solid (m.p. 208-210 °C). The structure (identified above) was confirmed by NMR.

Sodium borohydride (10% on alumina, 27.9 g, 73.5 mmole) was added to a suspension of Compound C

(15 g, 43.2 mmole) in ethyl acetate (400 ml). After stirring for one hour at room temperature, the reaction mixture was filtered and the alumina was washed with ethylacetate. The filtrate was freed of solvent to give 16 g of crude Compound D. The structure (identified above) was confirmed by NMR.

Ceric ammonium nitrate (75.3 g, 137 mmole) in water (150 ml) was rapidly added dropwise to a suspension of Compound D (16 g, 45.8 mmole) in acetonitrile (265 ml) and water (15 ml). After stirring two hours at room temperature, the reaction mixture was poured into ice/water (500 ml) and the precipitated product was collected, washed with water and air dried. The crude product was purified by chromatography (silica; dichloromethane/diethylether), and filtered to give 9.4g (64% yield) of Compound E (m.p. 219-221 °C). The structure (identified above) was confirmed by NMR.

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Preparation of Compound I:

Hydroxymethylquinone (2.23 g, 7 mmole), leuco methylene blue carbamyl chloride (2.44 g, 7 mmole) and 4-(N,N-dimethylamino)pyridine (0.85 g, 7 mmole) were dissolved in dichloromethane (50 ml) and cooled to 0 °C. 1,8-diazabicycloundec-7-ene (1.06 g, 7 mmole) was added, and the resulting mixture was stirred at 0 °C for 25 minutes. A small amount of acetic acid in water was added to neutralize the bases before diluting the mixture with ice water and dichloromethane. The organic phase was washed several times with cold water to remove methylene blue before drying over magnesium sulfate. The crude product was chromatographed on silica gel using 1% diethylether in dichloromethane as eluent. One gram of glassy product was obtained. Half of this product was slurried in diethylether/ethanol with two equivalents of concentrated hydrochloric acid to precipitate a gummy solid salt. This salt was slurried with a two-phase mixture of diethylether and water. The diethylether phase was removed and stripped to a solid (100 mg) Compound I. Thin layer chromatography demonstrated this solid to be reasonably pure. The resulting reducible compound having a leuco dye moiety was reasonably stable as a solid, but decomposed upon standing in a solution. NMR substantiated the structure. When contacted with ascorbic acid, an intense blue color was observed when the compound was warmed.

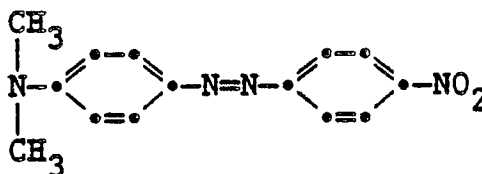
Example 2: Preparation of Compound II

Compound II identified above was prepared using the following procedure:

Preparation of Intermediate Compound:

A sample of the yellow dye (2.7 g, 0.01 mole)

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2,6-lutidine (2.14 g, 0.02 mole), 10% platinum on carbon (300 mg), a small stir bar, tetrahydrofuran (30 ml) and dichloromethane (70 ml) were added to a Parr bottle and subjected to 3 atmospheres of hydrogen pressure for about one minute until the color of the solution had faded from red to a pale yellow. The bottle was removed from the shaker under a nitrogen atmosphere and cooled to -78 °C with dry ice/isopropanol. A solution (10 ml) of a 1 molar solution of COCl₂ in toluene was added before warming the mixture to 0 °C for 20 minutes. The catalyst as well as some undissolved dye were removed by filtration before washing the solution with cold aqueous hydrochloric acid. After drying over magnesium sulfate, the solution was concentrated in vacuo, and diluted with a little diethylether to produce 1.2 g of solid carbamyl chloride. Thin layer chromatography showed the solid to contain some starting yellow dye. The solid was used directly in the next step.

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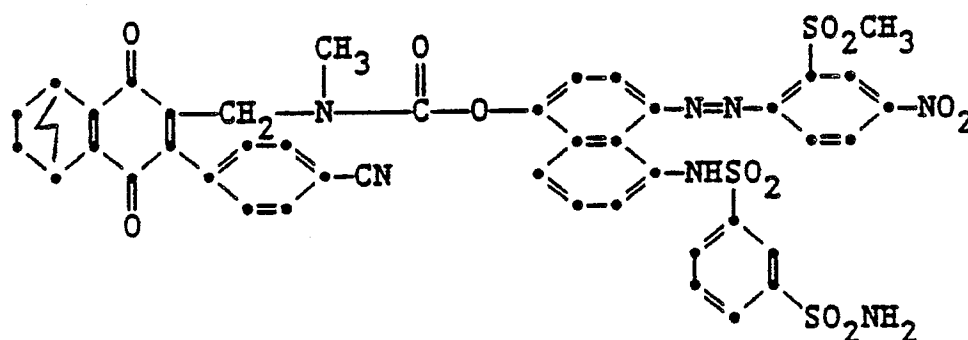
Preparation of Compound II:

Crude intermediate carbamyl chloride (1.2 g), hydroxymethylquinone (638 mg, 2 mmole), 4-(N,N-dimethylamino)pyridine (244 mg, 2 mmole) and 1,8-diazabicycloundec-7-ene (304 mg, 2 mmole) were reacted in 10 ml of dichloromethane as in Example 1. The procedure of Example 1 was followed to provide crude product which was chromatographed on silica using 4% diethylether in dichloromethane. After slurring in diethylether/methanol and then boiling acetonitrile, the eluted material was converted into 480 mg of yellow powder. NMR confirmed the structure of Compound II which was more stable than Compound I.

Example 3: Detection of Microorganism in Solution

This is a comparative example demonstrating the practice of the present invention to detect *E. coli* in an solution assay compared to a similar Control assay.

The reducible compound of the noted application used herein in the Control assay was



E. coli cells were cultured as described above. A dispersion of the Control reducible compound was prepared by dissolving the compound (16 mg) in N,N-dimethylformamide (1 ml) which had been acidified with 0.1% sulfuric acid. A sample (100 μ l) of this dispersion was added to TritonTM X-100 nonionic surfactant (200 μ l), and this second solution was added to potassium phosphate buffer (9.7 ml, pH 7.5) with stirring. A dispersion of the reducible compound II of this invention was similarly prepared except that 10.25 g of the compound was used.

Test solutions were prepared from the following: dispersion as described above (1.5 ml), glucose solution (25 μ l, 10% in water), trimethyl-1,4-benzoquinone ETA (0.01 molar in methanol, 25 μ l), potassium phosphate buffer (1.15 ml, 0.05 molar, pH 7.5) and *E. coli* cell suspension (final concentration of 5×10^5 cells/ml). Background solutions contained all reagents except microbial cells.

Optical densities were determined at 37 $^{\circ}$ C in a standard spectrophotometer at 650 nm for the Control assay and at 450 nm for the assay of this invention. The results are shown in Table I below as the change in optical density (Δ OD) after thirty minutes, corrected for background. The assay of this invention exhibited almost a 200% increase in cell response over the Control assay. This indicates a significantly greater sensitivity for the assay of this invention over the prior art assay.

TABLE I

Assay	Δ OD After 30 Minutes, 37 $^{\circ}$ C
Control	0.039
Example 3	0.114

Examples 4 and 5: Assays for Ascorbic Acid and NADH

These are comparative examples demonstrating the practice of this invention for the detection of ascorbic acid (Example 4) and NADH (Example 5) as compared to a Control assay using a reducible

compound as described in Example 3 above.

A dispersion of the Control reducible compound was prepared by dissolving it in N,N-dimethylformamide (16 mg/ml), followed by adding the resulting solution (100 μ l) to TritonTM X-100 nonionic surfactant (200 μ l). This solution was then added to a solution containing sodium phosphate buffer (9.3 ml, pH 7.5), 10% glucose solution (200 μ l) and trimethyl-1,4-benzoquinone or phenazine methosulfate ETA (0.01 molar). The phenazine methosulfate was used for detection of NADH while the benzoquinone was used for ascorbate detection. A dispersion of the reducible compound of this invention was similarly prepared except that 10.25 mg/ml was used.

Test solutions were prepared as follows: dispersion (1.5 ml) potassium phosphate buffer (1.4 ml, pH 7.5), and either ascorbate or NADH (100 μ l). A background solution contained all reagents except the analyte (ascorbate or NADH).

Optical densities were determined at 37° C using a standard spectrophotometer at 635 nm for the Control assay and at 500 nm for the assay of this invention. The results are shown in Tables II and III as the change in optical density (Δ OD) after 30 minutes and corrected for background. A substantial increase in response was seen for both assays of this invention over the Control assay.

TABLE II

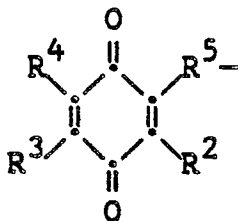
Assay	Analyte Concentration (molar)	Δ OD After 30 Minutes, 37° C
Control	3.3×10^{-6}	0.038
Example 4	3.3×10^{-6}	0.088
Control	3.3×10^{-7}	0.002
Example 4	3.3×10^{-7}	0.018

TABLE III

Assay	Analyte Concentration (molar)	Δ OD After 30 Minutes, 37° C
Control	3.3×10^{-6}	0.146
Example 5	3.3×10^{-6}	0.702
Control	3.3×10^{-7}	0.015
Example 5	3.3×10^{-7}	0.082

Claims

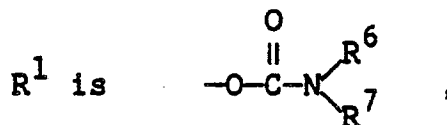
1. A reducible compound of the structure CAR-R¹ wherein CAR- is the quinone structure



- R² and R⁴ are independently hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted aryl or an electron withdrawing group,
 R⁵ is substituted or unsubstituted methylene, and
 R³ is the same as -R⁵-R¹, or is hydrogen, substituted or unsubstituted alkyl, substituted or unsubstituted

aryl or an electron withdrawing group, or R³ and R⁴, taken together, represent the atoms necessary to complete a substituted or unsubstituted fused carbocyclic ring, the compound characterized wherein

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R⁶ is substituted or unsubstituted aryl, and

R⁷ is substituted aryl, substituted or unsubstituted anilino, substituted or unsubstituted naphthylimino or an activated methylene group, or R⁶ and R⁷, taken together with the nitrogen atom to which they are attached, represent the carbon and hetero atoms which, after release of R¹ from said quinone nucleus and decarboxylation, form a leuco dye.

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2. The compound as claimed in claim 1 wherein at least two of R², R³ and R⁴ are independently electron withdrawing groups, or R³ and R⁴, taken together, represent the atoms necessary to complete a fused 5- to 7-member carbocyclic ring.

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3. The compound as claimed in either of claims 1 and 2 wherein R³ and R⁴, taken together, represent the atoms necessary to complete a fused 5- to 7-membered carbocyclic ring, and R⁵ is methylene.

4. The compound as claimed in claim 1 selected from the following group of compounds:

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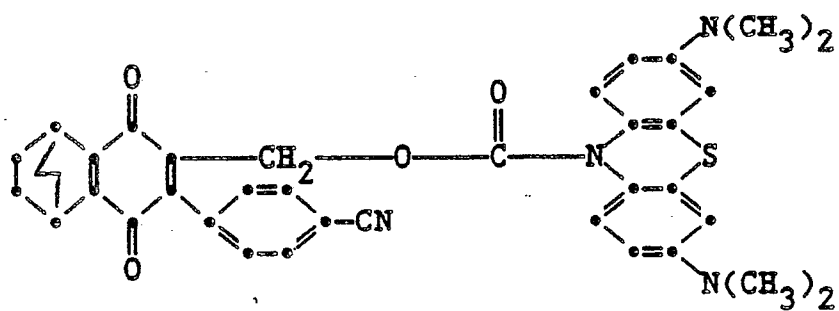
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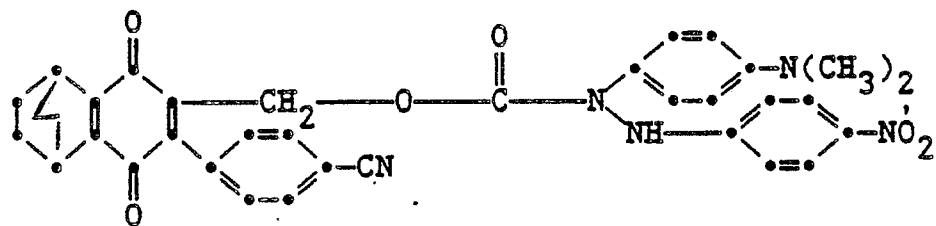
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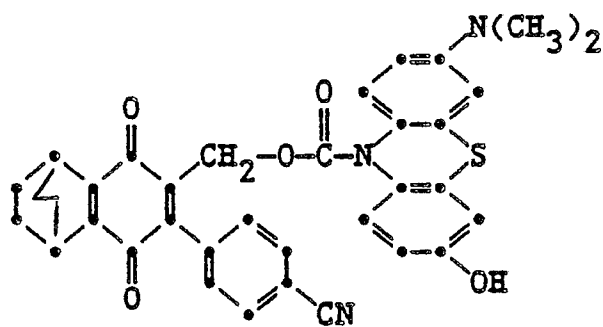
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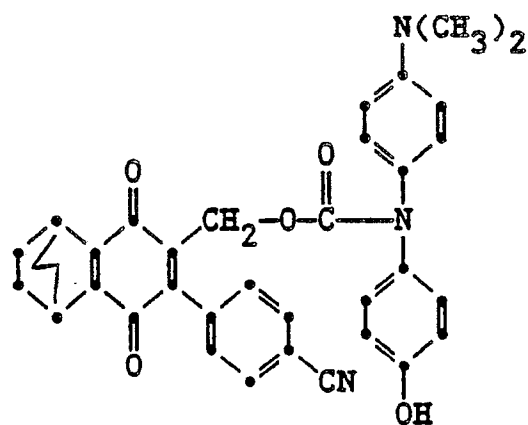
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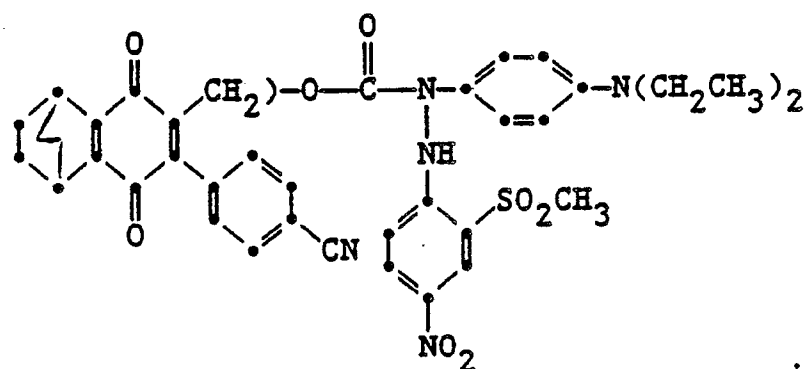
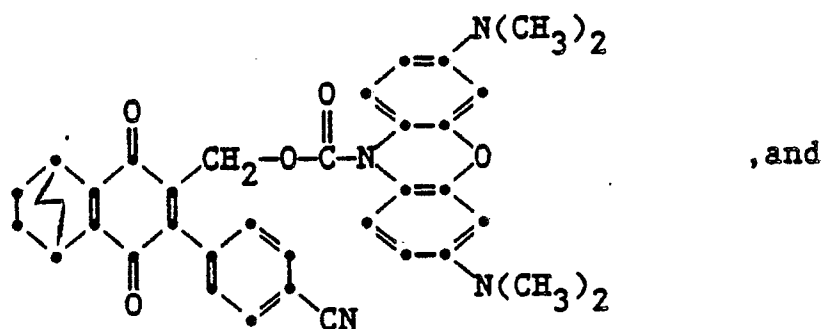
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25 5. An aqueous composition buffered at a pH of 9 or less and comprising a reducible compound as claimed in any of claims 1 to 4.

6. The composition as claimed in claim 5 further comprising an electron transfer agent.

30 7. A dry analytical element for the determination of an analyte comprising an absorbent carrier material and one or more reaction zones, the element also containing in one of the zones, a reducible compound as claimed in any of claims 1 to 4.

8. The element as claimed in claim 7 further comprising an electron transfer agent.

9. A method for the determination of an analyte comprising the steps of:

35 A. at a pH of 9 or less, contacting a sample of a liquid suspected of containing an analyte with a reducible compound as claimed in any of claims 1 to 4,

B. oxidizing the leuco dye with additional molecules of the reducible compound to provide a dye, and

C. detecting the provided dye as a result of the presence of the analyte which effects the release of R^1 from the reducible compound.

40 10. The method as claimed in claim 9 for the determination of living cells in the presence of an electron transfer agent.

11. The method as claimed in either of claims 9 or 10 for the determination of microorganisms in a urine sample.